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Constitutive CD8 Expression Allows Inefficient Maturation of CD4+ Helper T Cells in Class II Major Histocompatibility Complex Mutant Mice

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Summary

Although mature CD4+ T cells bear T cell receptors (TCRs) that recognize class II major histocompatibility complex (MHC) and mature CD8+ T cells bear TCRs that recognize class I MHC, it is possible that the initial commitment of an immature thymocyte to a CD4 or CD8 lineage is made without regard to the specificity of the TCR. According to this model, CD4+ cells with class I TCR do not mature because the CD8 coreceptor is required for class I MHC recognition and positive selection. If this model is correct, constitutive expression of CD8 should allow CD4+ T cells with class I–specific TCRs to develop. In this report, we show that mature peripheral CD4+ cells are present in class II MHC–deficient mice that express a constitutive CD8.1 transgene. These cells share a number of properties with the major class II MHC–selected CD4 population, including the ability to express CD40 ligand upon activation. Although mature CD4 cells are also detectable in the thymus of class II MHC mutant/CD8.1 transgenic mice, they represent a small fraction of the mature CD4 cells found in mice that express class II MHC. These results indicate that some T cells choose the CD4 helper lineage independent of their antigen receptor specificity; however, the inefficiency of generating class I–specific CD4 cells leaves open the possibility that an instructive signal generated upon MHC recognition may bias lineage commitment.

It is now clear that there is a connection between the recognition of MHC that promotes maturation of T cells (positive selection) and the commitment to a CD4 or CD8 T cell lineage. One manifestation of this connection comes from MHC-deficient mice. Mice bearing mutations that prevent expression of class II MHC contain very few mature CD4 T cells, whereas class I MHC–deficient mice express very few mature CD8 T cells (1–4). The mechanistic connection between MHC recognition by thymocytes and the CD4/CD8 lineage decision is still not understood. Two types of models have been put forth to explain the link between these two events. One model is that MHC recognition instructs or biases the decision to become a CD4 or CD8 cell such that intracellular signals generated upon class I MHC recognition lead to extinction of CD4 gene expression. Recognition of class II MHC would generate distinct intracellular signals that lead to the shut-off of CD8 gene expression (5, 6). An alternative to this "instructive" model is that the initial decision between the CD4 and CD8 lineage is made without regard to MHC specificity of the TCR. According to this "stochastic/selection" model, MHC recognition and positive selection occur after lineage commitment and thus, only thymocytes whose coreceptor and TCR recognize the same class of MHC molecule can mature (7, 8).

The stochastic/selection model predicts the existence of developmental intermediates bearing class I–specific TCRs that are committed to the CD4 lineage and cells with class II–specific TCR that are committed to the CD8 lineage which cannot complete maturation because they lack the appropriate coreceptor for MHC recognition. The analysis of thymic populations from MHC-deficient mice has revealed candidates for such developmental intermediates. A population of TCR+ $CD4^+CD8^{low}$ thymocytes is prominent in class II–deficient mice but not present in mice that are deficient for both class I and class II MHC (9, 10). An analogous TCR^+CD4^{low} $CD8^+$ population has also been reported in class I–deficient mice (11). Although the phenotype of these cells is consistent with the notion that they are transitional intermediates between double positive and single positive stages, there is no direct evidence that these cells can give rise to mature single positive T cells.

The stochastic/selection model also predicts that constitu-

tive coreceptor expression should permit the complete maturation of thymocytes that have chosen the wrong lineage. This prediction has been tested using CD8 and CD4 transgenic mice (12–15). In class I–deficient mice in which a CD4 transgene is expressed at 20× endogenous levels, a significant population of mature peripheral CD8 T cells is observed. A much smaller population of peripheral CD8 T cells is observed in class I MHC–deficient mice using a CD4 transgene expressed at more physiological levels. These results imply that some class I–specific T cells of the CD8 lineage are generated during thymic development and can be "rescued" by constitutive CD4 expression. In these experiments it is not possible to directly examine coreceptor downregulation in the thymus, because all thymocytes express the CD4 transgene.

In previous studies we have tested the predictions of the stochastic model using a constitutive CD8.1 transgene and two different class I–specific TCRs (12, 16). Although coexpression of the CD8.1 transgene with the F5 TCR leads to increased numbers of mature CD4 cells, coexpression of the CD8.1 transgene with the anti-HY TCR leads to increased numbers of mature CD8 cells but does not permit the development of mature CD4 cells. These different outcomes using two different individual TCRs prompted us to ask whether the rescue of class I–specific CD4 cells could be observed using a diverse TCR repertoire and physiological levels of CD8. In this study we show that expression of a constitutive CD8.1 transgene in class II MHC–deficient mice leads to the generation of a significant population of peripheral CD4 helper T cells. We also show that although mature $CD4^+CD8.2^-$ cells are detectable in the thymus of class II^-/CD8.1 transgenic mice, they represent only ~3% of the number of mature CD4 thymocytes found in mice that express class II MHC. We discuss these observations in terms of stochastic and instructive mechanisms for lineage commitment.

Materials and Methods

Transgenic Mice. Transgenic mice expressing the CD8.1 α and β transgenes (12) were backcrossed twice with mice homozygous for a targeted mutation in the class II, A_β^b allele (4), a kind gift of Laurie Glimcher, (Harvard School of Public Health and Medicine, Boston, MA). CD8.1 transgenic, class II MHC homozygous mutant offspring from the second backcross were identified by flow cytometry and sacrificed at 1–2 mo of age.

Cells. Thymocytes and lymph node T cells were obtained from whole organs which had been teased apart in cold M199 (Gibco, Grand Island, NY) supplemented with 5% FCS. Cell suspensions were passed through sterile nylon mesh. Lymph node cells were passed over plates coated with anti–mouse Ig as previously described (17) to remove B cells. Three-color analysis was performed using the following reagents: FITC-labeled anti-CD8.2 (2.43), PE-labeled anti-CD4 (GK1.5; Becton Dickinson & Co., Mountain View, CA), biotin-labeled anti-CD44, CD4 biotin, Tricolor®-labeled streptavidin (Caltag Laboratories, South San Francisco, CA), and PE-labeled anti-TCR-α/β (PharMingen, San Diego, CA). Data were collected on a FACScan® and FACS® 440 (Becton Dickinson & Co.).

Ligand Induction. For measurements of CD40 ligand induction, 3–4 × 10^6 lymph node T cells (B cell–depleted) were cultured for 5–6 h in 24-well plates in 1 ml of complete RPMI with PMA (10 ng/ml) and calcium ionophore (A23187, 500 ng/ml). Three-color analysis was performed using the following reagents: anti-Fc receptor (24G2; PharMingen), rat γ-globulin, Tricolor®-labeled streptavidin, FITC-labeled anti-CD8.2, and PE-labeled anti-CD4. CD40 ligand was detected using a fusion protein consisting of the extracellular domain of human CD40 and the constant region of human IgG1 (CD40–Ig) (18). CD40–Ig fusion protein was detected using biotinylated anti–human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Background staining was determined using duplicate samples in which the CD40–Ig fusion protein was left out. For nontransgenic and CD8.1 transgenic lymph node cells, 50,000 total events were collected. In the case of class II^- and class II^-/CD8.1 transgenic mice in which there were very few $CD4^+$ cells, data were also collected for gated $CD4^+CD8.2^-$ cells.

Results

To determine whether constitutive CD8 expression would permit the development of CD4 cells bearing class I–restricted TCRs, we crossed mice that are deficient for class II MHC due to targeted mutations in the I-A β gene (4) with mice bearing a constitutive CD8.1 transgene expressed at physiological levels (12). (In $H\text{-}2^b$ mice, class II I-E proteins are not expressed due to a mutation in the I-E α locus.) We then analyzed mice from the second backcross that were homozygous for the targeted class II MHC mutation and also carry the CD8.1 transgene (class II^-/CD8.1 transgenic). To determine whether mature CD4 cells are present in the periphery of these mice, we analyzed the lymph node T cells (B cell–depleted) by flow cytometry using monoclonal antibodies specific for CD4 and CD8.2 (endogenous CD8) (Table 1). Nontransgenic, CD8.1 transgenic mice, and class II MHC mutant mice that do not bear the CD8.1 transgene are included for comparison. In normal mice, the peripheral T cell population consists of $CD4^+CD8.2^-$ and $CD4^-CD8.2^+$ cells in roughly equal proportions. Expression of the CD8.1 transgene leads to a slight reduction in the mature $CD4^-CD8.2^+$ population but does not dramatically alter the proportions of the mature T cell subsets (12) (Table 1). In contrast, in mice that lack class II MHC, only ~3% of peripheral T cells are $CD4^+CD8.2^-$ (3, 4), reflecting the requirement for class II MHC recognition in the thymus for the maturation of these cells. Expression of the CD8.1 transgene in class II mutant mice leads to a significant increase in the number of peripheral $CD4^+CD8.2^-$ T cells, suggesting that CD8 expression rescues class I MHC–specific CD4 cells during development.

The few peripheral CD4 cells that are present in class II mutant mice differ from the bulk of the class II MHC–selected CD4 cells by a number of criteria, including the expression of the activation marker, CD44 (Pgp-1) (3). To determine whether peripheral CD4 cells in class II^-/CD8.1 transgenic mice resemble the atypical $CD4^+$ T cells found in class II mutant mice, we examined the expression of CD44 on peripheral CD4 cells (Fig. 1). Whereas most CD4 cells from normal and CD8.1 transgenic mice do not express CD44 (Figs. 1, *a* and *b*), CD4 cells in class II mutant mice are $CD44^+$ (Fig. 1 *c*). In contrast, the CD4 cells in class II^-/CD8.1 transgenic mice are predominately $CD44^-$ (Fig. 1 *d*) and, therefore, more closely resemble the major peripheral $CD4^+$ T cell population found in normal mice. Similar analysis for

Table 1. *Peripheral T Cell Subsets in Class II MHC Mutant, CD8.1 Transgenic Mice**

Mice	$CD4^+8.2^-$	$CD4^-8.2^+$	CD4/CD8 ratio
Nontransgenic	39	43	
	47	43	
	54	41	
	49	45	
	56	39	
	41	48	
	Mean: 48 (6.8)	43 (3.1)	1.1
CD8.1 transgenic	49	32	
	55	28	
	55	34	
	64	29	
	Mean: 56 (6.2)	31 (2.8)	1.8
Class II^-	2.4	81	
	3.3	79	
	2.7	90	
	2.2	89	
	2.6	87	
	2.2	88	
	Mean: 2.6 (0.4)	86 (4.5)	0.03
Class II^-/CD8.1 transgenic	11	65	
	13	71	
	13	77	
	15	77	
	18	54	
	8.1	78	
	Mean: 13 (3.4)	70 (9.4)	0.19

* Lymph node T cells were stained with antibodies against CD4 and CD8.2 (endogenous CD8) as described in Materials and Methods. Each line represents the values from an individual mouse. Values are percentage of lymph node T cells in each subset, and standard deviations are in parentheses.

cell surface expression of heat-stable antigen (HSA)[1], class I MHC K^b, NK1.1, CD45RB, Vβ6, and Vβ8 revealed no significant difference between the rescued CD4 cells and the major CD4 population of normal mice (data not shown).

In normal mice, helper activity is associated with class II MHC-selected $CD4^+$ T cells. Because the $CD4^+8.2^-$ cells in class II^-/CD8.1 transgenic mice resemble normal CD4 cells but were presumably selected on class I MHC, it is unclear whether these cells would exhibit helper or cytotoxic T cell effector functions. To investigate this question, we examined the ability of peripheral $CD4^+$ cells to express the

[1] *Abbreviation used in this paper:* HSA, heat-stable antigen.

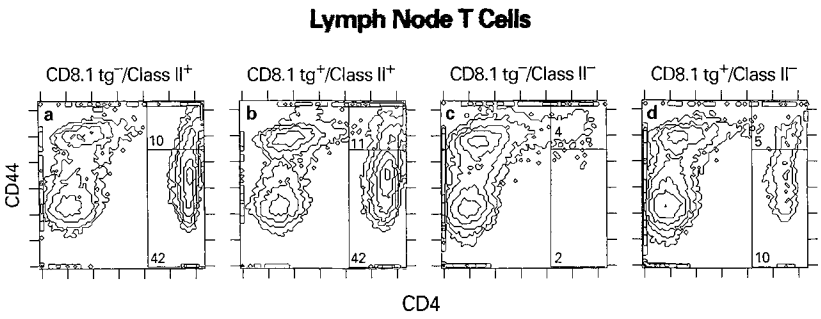

Figure 1. CD44 and CD4 expression on lymph node T cells from class II^-/CD8.1 transgenic mice. Lymph node T cells (B-depleted) were stained for CD4 and CD44 (Pgp-1) as described in Materials and Methods. (*a*) Nontransgenic; (*b*) CD8.1 transgenic; (*c*) class II^-; and (*d*) class II^-/CD8.1 transgenic.

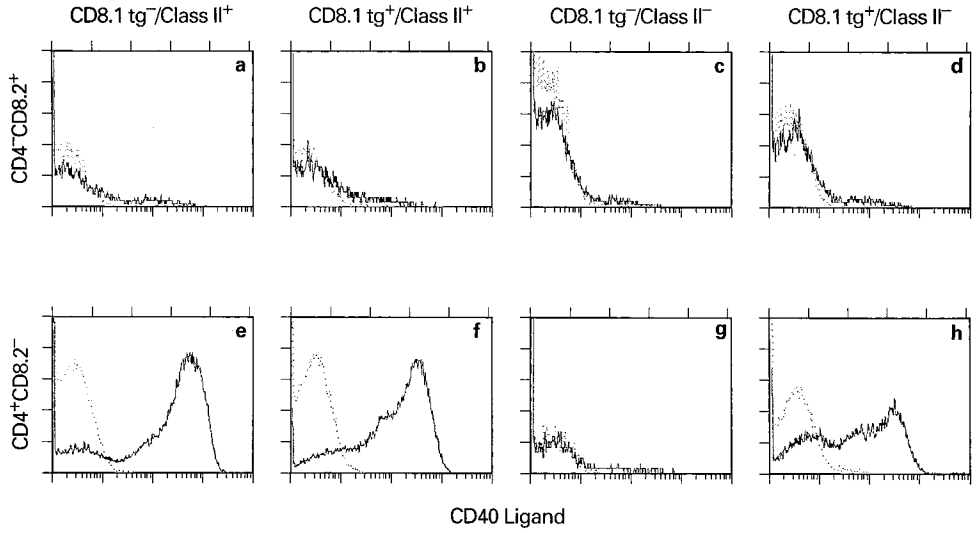

Figure 2. CD40 ligand expression on activated T cells. (*a–d*) CD4⁻CD8.2⁺ cells; (*e–h*) CD4⁺ CD8.2⁻ cells. Lymph node T cells (B-depleted) were stimulated with PMA and ionomycin and stained for CD4, CD8.2 (endogenous CD8), and CD40–Ig as described in Materials and Methods. Dotted lines represent background staining from parallel samples in which CD40–Ig was omitted.

ligand for CD40. CD40 is a receptor expressed by B cells, and the CD40 ligand is expressed preferentially by CD4⁺ T cells after activation by PMA and ionomycin (19, 20). CD40 ligand was detected using a fusion protein consisting of the extracellular domain of human CD40 and the constant region of human IgG1 (18). Lymph node T cells from class II⁻/CD8.1 transgenic mice and control mice were exposed to PMA and ionomycin for 5–6 h and analyzed by three-parameter flow cytometry using monoclonal antibodies against CD4, CD8.2, and the CD40–Ig fusion protein (Fig. 2). Stimulated CD4⁺8.2⁻ cells from normal mice express a high level of CD40 ligand (Fig. 2 *e*), whereas activated CD4⁻8.2⁺ cells express very little CD40 ligand (19, 20) (Fig. 2 *a*). Likewise, in CD8.1 transgenic mice, CD40 ligand is induced on CD4⁺ T cells, indicating that expression of transgenic CD8.1 does not significantly impair their ability to express CD40 ligand (Fig. 2 *f*). CD4⁺ cells from class II MHC-deficient mice do not express significant levels of CD40 ligand after PMA and ionomycin treatment (Fig. 2 *g*), consistent with indications that they differ from the majority of class II MHC-selected CD4⁺ T cells. In contrast, the CD4⁺8.2⁻ cells from class II⁻/CD8.1 transgenic mice do express significant levels of CD40 ligand upon exposure to PMA and ionomycin (Fig. 2 *h*). These results indicate that

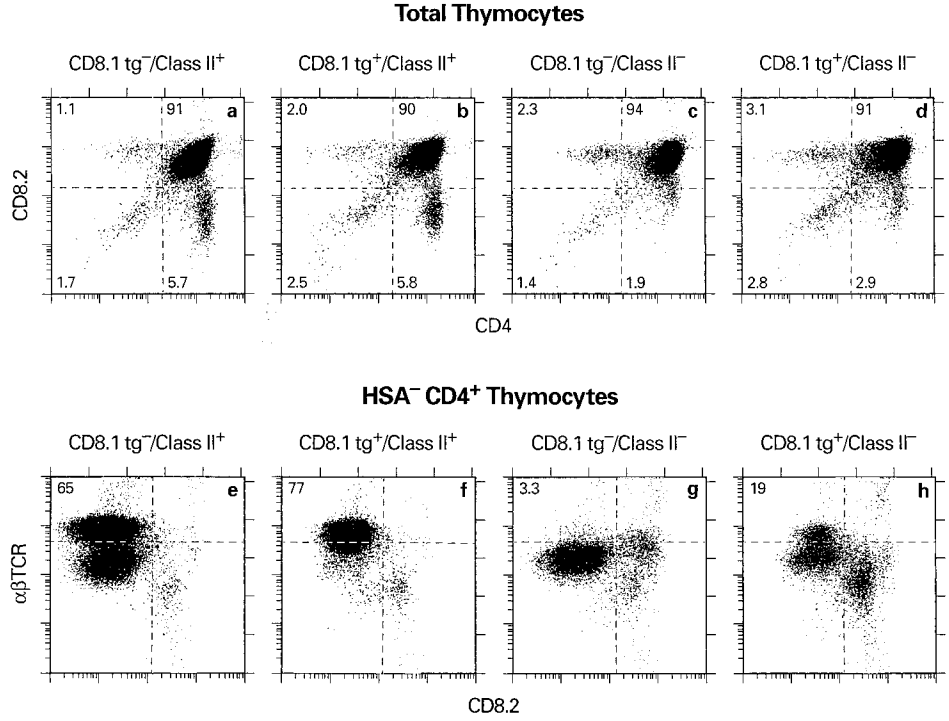

Figure 3. Thymocytes from (*a* and *e*) nontransgenic, (*b* and *f*) CD8.1 transgenic, (*c* and *g*) class II⁻, and (*d* and *h*) class II⁻/CD8.1 transgenic mice. (*a–d*) CD4 and CD8.2 expression on total thymocytes, (*e–h*) TCR-α/β and CD8.2 expression on CD4⁺HSA^{low} thymocytes. CD8.1 and class II⁻/CD8.1 transgenic mice have on average two- to threefold fewer total thymocytes than nontransgenic and class II⁻ mice.

although these cells are presumably selected on class I MHC, they respond to stimulation in a fashion similar to that exhibited by normal $CD4^+$ T helper cells.

Although substantial numbers of mature CD4+ T cells are found in the periphery of class II^-/CD8.1 transgenic mice, we considered the possibility that these cells might actually be generated inefficiently in the thymus and accumulate over time in the periphery. To assess the efficiency of generating the rescued CD4 cells, we examined thymic populations of class II^-/CD8.1 transgenic mice (Fig. 3 and Table 2). Quantitation of mature $CD4^+CD8.2^-$ cells in the thymus is complicated by the heterogeneity of thymic T cells. If other markers are assessed, there are at least three distinct populations within the $CD4^+CD8.2^-$ quadrant defined in Figs. 3 *a–d*.

To accurately quantitate mature $CD4^+CD8.2^-$ thymocytes, we enriched for these thymocytes by depleting cells expressing HSA. We then analyzed the resulting population for expression of CD4, CD8.2 and TCR-α/β (Table 2 and Figs. 3, *e–h*). In HSA^- thymocytes of nontransgenic mice (Fig. 3 *e*), two $CD4^+CD8.2^-$ populations are apparent: one that expresses high levels of TCR-α/β and represents the precursors of peripheral $CD4^+$ T cells, and one that expresses intermediate levels of TCR. The latter population may contain cells in transition between the immature $CD4^+$ $CD8^+$ "double positive" and the mature $CD4^+CD8^-$ "single positive" stages (9, 21, 22). In addition, HSA^- thymocytes also contain a subset termed "thy0" cells by Hayakawa et al. (23) that resembles the $CD4^-CD8^-$ TCR^+-α/β thymocytes in a number of respects. For example, both have

Table 2. *Thymic Subsets in Class II MHC Mutant, CD8.1 Transgenic Mice**

Mice	$CD4^+/CD8.2^-TCR^{high}$	$CD4^-CD8.2^+TCR^{high}$	CD4/CD8 ratio
Nontransgenic	23	17	
	42	11	
	48	17	
	35	27	
	28	8.0	
	Mean: 35 (10)	16 (7.3)	2.2
CD8.1 transgenic	35	13	
	40	23	
	49	26	
	33	15	
	Mean: 39 (7.1)	19 (6.2)	2.1
Class II^-	0.5	38	
	0.7	62	
	0.9	59	
	1.0	80	
	0.7	87	
	0.6	41	
	0.6	35	
	Mean: 0.7 (0.2)	57 (21)	0.012
Class II^-/CD8.1 transgenic	2.9	49	
	6.7	67	
	3.5	56	
	4.7	81	
	4.6	84	
	6.5	62	
	2.8	39	
	4.8	40	
	Mean: 4.6 (1.5)	60 (17)	0.077

* HSA-depleted thymocytes were stained with antibodies against CD4, CD8.2 (endogenous CD8), and TCR-α/β as described in Materials and Methods. Representative data are shown in Fig. 3. Each line represents the values from an individual mouse. Values are percentage of HSA^- thymocytes in each subset, and standard deviations are in parentheses.

an unusually high usage of Vβ8 and the majority express CD44, NK1.1, Ly-6C, and intermediate levels of TCR (23–27; Coles, M., and D. Raulet, manuscript submitted for publication). When compared with nontransgenic mice (Fig. 3 *e*), mature thymocytes from CD8.1 transgenic mice contain normal numbers of the $CD4^+CD8.2^-TCR^{high}$ cells, although the TCR intermediate population is significantly reduced (Fig. 3 *f*). As expected, $CD4^+CD8.2^-TCR^{high}$ cells are undetectable in class II^- mice, although the TCR intermediate population is still present (Fig. 3 *g*). In the class II^-/CD8.1 transgenic mice (Fig. 3 *h*) there is a small but detectable population of $CD4^+CD8.2^-TCR^{high}$ thymocytes. It is likely that these cells are the precursors of the peripheral CD4 cells and were selected using class I–specific TCR and transgenic CD8.1.

How does the size of the mature $CD4^+CD8.2^-TCR^{high}$ population in class II^-/CD8.1 transgenic mice compare with the corresponding population in the thymus of mice which express class II MHC? When considered as a fraction of HSA^- thymocytes, the $CD4^+CD8.2^-TCR^{high}$ population is 13–15% of that found in wild-type mice or CD8.1 transgenic mice (Table 2); however, because the HSA-depleted thymocytes from different mice contain variable numbers of thy0 cells and residual immature cells, the extent of rescue by the CD8.1 transgene might best be estimated by comparison to the mature CD8 population. Because the proportion of $CD4^-CD8.2^+TCR^{high}$ cells shows only minor variations from sample to sample and does not differ consistently in the different mouse strains examined (Fig. 3 and data not shown), this population should serve as a valid denominator. In Table 2, the number of $CD4^-CD8.2^+TCR^{high}$ and $CD4^+CD8.2^-TCR^{high}$ thymocytes and the CD4/CD8 ratios are presented. The class II^-/CD8.1 transgenic mice have a CD4/CD8 ratio of 0.08 compared with 2.2 in normal mice. According to this measure, the rescued CD4 cells in class II^-/CD8.1 transgenic mice represent 3% of the mature CD4 cells found in the thymus of nontransgenic mice. Thus, although rescued CD4 cells are clearly detectable in the thymus, they represent a small fraction compared with the class II MHC–selected CD4 cells found in normal mice.

Discussion

Thymic development leads to the generation of $CD4^-8^+$ cells with class I–specific TCRs and $CD4^+CD8^-$ T cells with class II–specific TCRs. It has been suggested, however, that the commitment to a CD4 or CD8 lineage may initially be made without regard to the MHC specificity of the TCR and that the subsequent requirement for coengagement of the appropriate coreceptor (CD8 for class I MHC or CD4 for class II MHC) would ensure that only T cells whose TCR and coreceptor recognize the same class of MHC molecules will mature (7, 8). To test this possibility, a number of investigators have asked whether constitutive coreceptor expression could permit the development of mature T cells expressing the wrong endogenous coreceptor. Thus far, this approach has yielded mixed results. Whereas expression of a constitutive CD4 transgene allows the development of CD8 cells bearing a class II MHC–specific transgene-encoded TCR (15), analogous experiments using constitutive CD8 transgenes lead to the appearance of mature $CD4^+$ T cells with one class I–specific TCR (16) but not another (12–14). Moreover, although peripheral CD8 cells are present in class I–deficient /CD4 transgenic mice, there are only about 5% of the number of mature CD8 cells found in normal mice when the CD4 transgene is expressed at physiological levels. In this report we determine the efficiency of rescue of mature $CD4^+$ class I–specific T cells with a diverse TCR repertoire. We show that expression of the CD8.1 transgene in class II MHC-deficient mice leads to a significant increase in mature peripheral $CD4^+$ T cells. In these experiments, the ability to distinguish endogenous CD8 from transgenic CD8 makes it possible to assess coreceptor downregulation directly in the thymus and, thus, estimate the efficiency of generating these mature CD4 cells. Such an analysis revealed that although fully mature CD4 cells could be detected in the thymus, they represent a small fraction of the mature CD4 population found in the thymus of normal mice. These results lend further support for the presence of a stochastic mechanism in the development of CD4 and CD8 T cells; however, the low efficiency of generating CD4 cells in these mice implies either that the stochastic mechanism does not represent a major developmental pathway or that the rescue of these cells by the CD8 transgene is extremely inefficient.

In normal mice, helper activity is predominantly associated with class II–selected, $CD4^+$ cells, whereas the majority of class I–selected, $CD8^+$ T cells display cytotoxic activity. This bias in effector function could result from a coordinate expression of genes involved in effector function with the CD4 or CD8 genes. Alternatively, the bias could be due to intracellular signals generated by MHC recognition and might be independent of coreceptor gene expression. Our data support the former possibility. We find that mature class I–selected, $CD4^+$ T cells in class II^-/CD8.1 transgenic mice express CD40 ligand upon activation, similar to the class II–selected $CD4^+$ T cells in normal mice. This implies that the choice to express helper or killer effector functions is made independently of MHC recognition and correlates with endogenous coreceptor gene expression.

Models to explain the connection between positive selection and CD4/CD8 lineage commitment were initially formulated based on the assumption that positive selection was driven by a single discrete recognition event, an assumption that is challenged by recent experiments. A comparison of thymic subsets in class I MHC–deficient and class I and class II MHC double-deficient mice led to the suggestion that recognition of class I MHC allows partial maturation of CD4-committed thymocytes but that class II MHC is required to complete their development (9, 10). This implies that the process of positive selection might normally involve multiple or prolonged encounters with MHC. This possibility is also supported by observations that both class I and class II MHC can participate in the selection of T cells bearing individual transgene-encoded TCRs (Fowlkes, B. J., A. Itano, and E. Robey, unpublished observations).

It is worth reexamining the possibility that the CD4/CD8 lineage decision may be driven by instructive signals, given the inefficiency of rescue and in light of indications that positive selection may not occur in a single step. For example, it is possible that an initial encounter with MHC generates an instructive signal and that this signal biases, but does not dictate, lineage commitment (6). According to this scenario, a $CD4^+CD8^+$ thymocyte expressing a class I–specific TCR might have a 95% probability of becoming a CD8 cell and a 5% probability of choosing the CD4 lineage after an initial encounter with class I MHC. Because the cell must continue to recognize MHC after it turns off expression of one coreceptor, the 5% that turned off CD8 would not complete maturation. In this two-step instruction model, one MHC recognition event serves to bias lineage commitment, while a second ensures that cells that choose the wrong lineage do not become fully mature. Constitutive coreceptor expression might serve to override this back-up mechanism and allow those few cells of the wrong lineage to develop. At first glance, the presence of significant numbers of $CD4^+CD8^{low}$ cells that appear to be in transition to the CD4 single positive stage in class II mutant mice argues against this scenario; however, these cells might be generated inefficiently and might accumulate in the thymus over time.

It is also possible to account for the inefficient generation of $CD4^+$ class I–specific T cells by a purely stochastic model. For example, one could argue that the choice between the CD4 and CD8 lineages is totally independent of MHC specificity of the TCR, but that the CD8 transgene does not function normally in a thymocyte that has chosen the CD4 lineage. If the presence of CD4 inhibits CD8 function, then CD8 might become more active as thymocytes turn off CD4. In this case, the CD8 transgene might not be fully functional in a thymocyte that still expresses CD4. A definitive resolution of the mechanism of CD4/CD8 lineage commitment awaits a better understanding of the role of CD4 and CD8 in thymic development and the lineage relationship between thymic subsets.

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